INFLUENZA STUDIES

III. ATTEMPTS TO CULTIVATE FILTRABLE VIRUSES FROM CASES OF INFLUENZA AND COMMON COLDS *

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During the winter of 1919-20 we attempted to cultivate filtrable viruses from certain respiratory infections, employing the technic of Foster,[1] and in addition, other methods that seemed promising. Fifty-five samples of nasopharyngeal secretions from 44 individuals have been studied, 38 of the samples being from common colds, 9 from influenza, and 8 from normal persons. Most of the colds were simple acute rhinitis, 7 were accompanied by a bronchitis, 1 by pharyngitis, 2 by sinusitis, 1 by tonsillitis, and 1 proved to be a nasal diphtheria. The time of collecting these samples after the period of onset of the disease is shown in table 1.

TABLE 1

TIME AFTER ONSET WHEN SAMPLES WERE COLLECTED

	Days							
	1	2	3	4	5	5 After 6th		
Colds	11	2	8	8	—	—	9	38
Influenza	—	1	2	2	1	1	2	9
Normal	—	—	—	—	—	—	—	—
Total	11	3	10	10	1	1	11	55

In collecting the samples, the nasopharynx was washed out with 50-75 c c of warm Ringer's solution into a sterile glass or rubber stoppered bottle containing glass beads. Five of the samples stood overnight in an icebox, but the others were handled promptly, in most cases within 2 hours of collection.

In every case a loopful of B. prodigiosus was added to the sample before filtration; it was then shaken thoroughly with the glass beads, and a plain agar slant made to show that the organism was present.

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[1] J. Infect. Dis., 1917, 21, p. 451.

Then the sample was passed through a Mandler 6 x 1.5 cm. filter, under a vacuum pressure of from 14 to 60 cm. of mercury, the time of filtration being from 5 to 30 minutes. The freedom of the filtrate from B. prodigiosus and other bacteria was determined by inoculating 5 c c of it into about 20 c c of glucose broth. This test for permeability was made each time because often a previously impermeable filter will suddenly allow organisms to pass through.

The medium used most was the tissue-ascitic fluid employed by Foster [1] and by Flexner and Noguchi.[2] About 15 c c of ascitic fluid were put into culture tubes 20 x 1.5 cm., a piece of fresh sterile rabbit kidney was added, and a 4 cm. layer of mineral oil, autoclaved at 20 pounds for 20 minutes on 2 successive days, was poured on top. These tubes were incubated at 37 C. for several days to determine their sterility. At first the blood in the rabbit kidney laked and formed a clear rosy zone around the tissue. After a few days this faded and the pink tissue, gradually became gray.

Other mediums used were:

1. Equal parts of ascitic fluid and plain 2% agar poured into a tube over a piece of sterile rabbit kidney and covered with a layer of sterile mineral oil.

2. One part of horse serum, inactivated at 56 C. for 30 minutes to ten parts of 2% agar, with sterile rabbit kidney and a layer of oil.

3. Noguchi's [3] serum water, made with horse serum, under oil.

4. Glucose broth with sterile rabbit kidney.

5. A modification of Noguchi's serum water, without the sterile tissue, and with 0.5% peptone and 1% glucose, the P_H value adjusted to 8.

6. A plain 1% glucose meat infusion broth with 2% peptone, and 0.5% NaCl.

7. Serum-glucose broth, with 1 part of horse serum to 4 of broth.

8. Ascitic-glucose broth, with 1 part of ascitic fluid to 1 of broth.

The first 4 mediums were incubated 48-72 hours to determine their sterility; the last 4 were boiled vigorously just before using to expel oxygen, and, in the case of the serum glucose broth and the ascitic glucose broth, the ascites and serum were added at the time of inoculation of filtrate. A clear mineral oil was used as the anaerobic seal

[2] J. Exper. Med., 1913, 8. p. 461.
[3] Ibid., 1911. 14, p. 99.

in addition to the considerable depth of fluid in the first 3 mediums above mentioned; in the last 5, anaerobiosis was secured by means of a marble seal in the constricted tube designed by one of us.[4] The agar and glucose broth used in these mediums had a veal infusion basis, and the reaction was P_H 8.

The mediums were inoculated by means of sterile graduated pipets, with an amount of filtrate varying from 0.5 c c to 10 c c. These cultures, with the uninoculated controls accompanying them in each case, were incubated at 37 C. Not every kind of medium was used for all the samples, but each was used a number of times, as shown in table 2.

TABLE 2

DISTRIBUTION OF MEDIUMS IN 55 SAMPLES

Kind of Medium	Times Used
Ascites-kidney	44
Ascites-kidney-agar	12
Serum-kidney-agar	17
Noguchi's serum water	23
Glucose-kidney broth	26
Modified Noguchi's serum water	20
Plain glucose broth	48
Serum- or ascites-glucose broth	33

With 13 sets of cultures, a growth of B. prodigiosus appeared in the inoculated tubes within 72 hours, showing that the filters used were permeable to bacteria of that small size. In 3 others a quick growth of the hay bacillus, and in one of Staph. albus, indicated some fault of technic. The other 38 sets of cultures were incubated for from 3 to 6 weeks, and were carefully observed from day to day. In all of the ascitic-tissue tubes, after a few days' incubation, a faint haze appeared around the tissue. This usually spread slowly upward, always being sharply demarcated from the clear fluid above. Sometimes this haze was more pronounced in the inoculated tubes; often no difference could be seen between them and the uninoculated ones; and sometimes the cloudiness was more marked in the control tubes. Aerobic subcultures on blood agar and glucose broth were made from material pipetted from these hazy zones. Where the haziness was pronounced, these subcultures often showed bacterial growth, the most common organisms being Staph. citreus, a tiny plump gram-positive bacillus, and diphtheroid bacilli. Occasionally other cocci were found. The source of these organisms, which appeared as late contaminations

[4] Univ. Calif. Pub. in Path., 1915, 2, p. 147.

in the tissue-ascitic fluid culture tubes, remains unexplained. The nature of the organisms, their late appearance, often after more than two weeks' incubation, and their frequent occurrence in controls as well as in inoculated tubes, all seem to indicate that they were not acquired during filtration and that they were not due to faulty technic. Perhaps they were the result of organisms deeply lodged in the kidney tissue, which required a long time to grow to the surface.

Twenty-three sets of ascitic-tissue tubes were incubated with no bacterial contamination for from 3 to 6 weeks, until discarded. Subcultures made from them into the same medium from time to time, and the controls as well, soon took on the characteristic appearance, with the sharply demarcated haze around the tissue; but those made into the solid medium, such as ascitic-tissue agar and serum-tissue agar, showed little change. A haziness in the tubes which contained no tissue always meant bacterial contamination, which could be demonstrated by strains and subcultures on agar and in broth. In all mediums containing tissue there was some haziness after a time, due apparently to autolysis of the tissue.

Giemsa, Gram, and Loeffler's methylene blue stains were made at intervals, beginning with the seventh day, from all cultures and subcultures, using material withdrawn from around the tissue with a sterile capillary pipet. With the Giemsa stains, the methods used by Foster and by Noguchi were carefully followed. The films were air-dried, fixed one hour in methyl alcohol, stained overnight in a jar containing one drop of Giemsa stain to every 1 c c of distilled water, immersed in acetone a few seconds to remove the excess stain, and then washed in distilled water and dried. With Gram's method, safranin was used as a counterstain.

Microscopically these stains from the ascitic-tissue cultures presented a puzzling picture. They showed, among the débris, a great many tiny round bodies of various sizes, purple with Giemsa's stain, and gram-positive. There was little uniformity or regularity about them. These bodies often strikingly resembled tiny cocci, and, while usually occurring singly, were often in pairs and groups, and occasionally in short chains. Similar bodies seemed to be equally numerous in the stains made from control tubes, and also in plain ascitic fluid. They were particularly abundant in the Gram stains, especially if the materials used had not been freshly filtered, or if the slides were not very clean.

They were found in abundance in all mediums containing autolyzed tissue. To a lesser extent they were present on stains made from the cultures and controls in the other mediums used after incubation for a time, and finally they were found on stained blank slides. There were no differences noted in the slides made from cases of colds or influenza, or from normal persons.

These bodies might have been considered the globoid bodies described by various investigators, but we were unable to convince ourselves that they were other than artefacts derived on the one hand from the disintegration of the tissues added to the mediums, or on the other, from precipitates in the mediums and stains employed. The necessity of great caution in interpreting microscopic findings from such "cultures" is well illustrated in the extraordinary retraction made recently by certain English investigators.[5] We consider our results totally negative so far as the cultivation of a filtrable virus from our material was concerned.

In order to be sure that these negative results were not due to the unsuitability of the mediums, and that it was perhaps justifiable to assume that filtrable viruses ought to grow in them, some of each type of medium was used for the cultivation of certain strict anaerobes and especially delicate facultative aerobes. B. sporogenes, B. botulinus, B. welchii, B. tetani, B. chauvæi, and Vibrion septique all grew well during 48 hours' incubation. The freedom of these cultures from aerobic contamination was checked by a plain agar slant. Streptococcus viridans grew well in all of the mediums; B. influenza grew in all containing tissue and with special luxuriance in the tissue-ascitic fluid; Diplococus gonorrhœæ grew in all except the glucose broth, and also showed a special preference for the tissue-ascitic fluid. Except in the first few trials, every new lot of medium was tested by growing all of these organisms in it.

DISCUSSION

The methods used by Foster with colds have been followed as closely as possible. Gibson, Bowman, and Conner [6] followed Foster's technic in their work with influenza, and reported positive results. Our attempts to repeat this work have been unsuccessful. After one trial we did not attempt to employ the modified medium of Wilson,[7] both

[5] Brit. Med. Jour., 1919, 2, p. 233.
[6] Brit. Med. Jour., 1918, 1, p. 645; 1919, 2, p. 331.
[7] Ibid., 1919, 1, p. 602.

on account of the technical difficulty of preparing it without contamination, and because there was no way in which, by preliminary incubation, freedom from bacterial contamination could be assured.

Noguchi, Foster, and others stated that all samples of ascitic fluid were not suitable for the growth of the organisms which they found. The only criterion seems to have been whether or not their cultures grew in it. This test it was obviously impossible for us to apply. However, it seems that the decided preference shown by very delicate bacteria for the mediums containing samples of the ascitic fluid used, and especially for the plain ascites-tissue, is a criterion of some value. That the conditions of anaerobiosis were fulfilled is proved by the ready growth of the strict anaerobes used.

The first sample of ascitic fluid became contaminated in the laboratory and had to be filtered to insure its sterility. The samples used later were originally sterile, free from bile color, and formed a loose fibrin in the culture tubes. These three qualities Noguchi emphasized as being highly desirable, and in the mediums made with these later samples the bacteria tested grew with great luxuriance.

With the idea that perhaps a possible disadvantage of the Foster-Noguchi medium lay in the fact that there was no suitable way of removing oxygen just before use, several kinds of mediums were used that could be boiled out, using the mechanically sealed constricted tubes referred to above. These furnished ideal conditions of anaerobiosis. A possible error lay in the addition of glucose to such mediums, for no matter how carefully a reaction is adjusted, the presence of glucose is always a potential source of acid. Both Noguchi [8] and Foster [1] stated that the medium they used was slightly alkaline, though the degree of alkalinity and the indicator used were not mentioned.

SUMMARY

These experiments offer no evidence in support of the theory that the cause of either common colds or influenza is a filtrable virus.

In attempting to cultivate filtrable viruses from the nasopharyngeal secretions in colds and influenza, no bodies were found in the "cultures" which could not be found also in those from normal persons, in controls in all simple mediums examined, and on blank slides.

[8] Jour. Exper. Med., 1912, 16, p. 199.

It is recognized that negative experiments, limited to the attempted cultivation of a filtrable virus, and including no attempts to reproduce the disease in animals, do not offer conclusive evidence that such a virus is not involved.

No conclusions can be drawn concerning influenza, on account of the few cases examined, together with the fact that samples of such were not collected during the earliest stages of the disease. However, the uniformly negative results obtained with a large and representative number of colds are not without significance.